Formation and properties of C1-inhibitor polymers

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Received 2 May 1995; revised version received 31 May 1995

Abstract Heating of the serpin C1-inhibitor above 55°C induced the formation of inactive polymers. Western blotting of non-denaturing gels showed that the polymers bound to the conformation specific monoclonal antibody 4C3, suggesting that a similar conformational change to that occurring in complexed or cleaved inhibitor had taken place. N-Terminal analysis of tryptic peptides which bound to 4C3 showed that the epitope resides within residues 288–444, a region which includes parts of β-sheets A and C. α_1-Antichymotrypsin, α_2-antiplasmin, angiotensinogen and thyroxine binding globulin also polymerised on heating, indicating that this is a property of many serpins.

Key words: Serpin; C1-inhibitor; Complement; C1s; Monoclonal antibody

1. Introduction

The serpins are a diverse family of proteins, many of which are serine proteinase inhibitors (for example, C1-inhibitor, antithrombin, α_1-proteinase inhibitor and PAI-1), although some have no known inhibitory function (for example, ovalbumin) [1]. A characteristic of serpins is that the native molecule is not the most stable form but a kinetically trapped metastable intermediate [2]. Also, the serpin reactive centre loop region which binds to the proteinase is able to adopt a variety of conformations [3]. One of these conformational states involves the interaction of the reactive centre loop with β-sheet C of an adjacent molecule, which can lead to polymer formation.

Polymer formation has been implicated in the pathological states associated with the α_1-proteinase inhibitor Z, and the antithrombin Rouen-VI, variants [4–6]. Variants of C1-inhibitor which readily polymerise have also been reported [7,8]. Following reaction with target proteinases normal plasma C1-inhibitor undergoes a conformational change detected by a monoclonal antibody which can bind complexed or reactive centre loop cleaved C1-inhibitor [9,10]. We report here that normal plasma C1-inhibitor readily polymerises on heating, and that these polymers bind to this monoclonal antibody.

2. Materials and methods

C1s was from Enzyme Research Laboratories (South Bend, IN) and C1-inhibitor was provided by Dr. E.P. Pâques (Behring, Marburg, Germany). Both were assayed as described previously [11]. C1-inhibitor was run over a column of Sepharose-CL6B (Pharmacia, Piscataway, NJ) prior to use to remove any polymers which spontaneously form on storage. Angiotensinogen, α_2-antiplasmin and α_1-antichymotrypsin were from Athens Research (Athens, GA). Thyroxine binding globulin was from Calbiochem (San Diego, CA). TPCK-treated bovine trypsin was from Worthington (Freehold, NJ) and was active site-titrated with para-nitrophenol guanidinobenzoate [12]. Monoclonal antibodies 3C7 and 4C3 were prepared as described previously [9].

For qualitative comparison of the effect of trypsin on native and heated C1-inhibitor (Fig. 2), the C1-inhibitor samples (5 μg) were incubated with trypsin (0.1 μg) in phosphate-buffered saline at 23°C for the indicated times, followed by addition of phenyl methyl sulphonyl fluoride (PMSF) to 0.5 mM and dichloroisocoumarin (DCI) to 0.8 mM and SDS-PAGE [13] sample buffer. For 4C3 epitope analysis, native C1-inhibitor (0.35 mg) was digested with trypsin (0.07 mg) in 170 μl of 29 mM Tris-HCl, pH 8.3, 12 mM calcium chloride, for 30 h at 37°C. The reaction was stopped with 170 μl of SDS-PAGE sample buffer containing 2% (v/v) β-mercaptoethanol. Peptides capable of binding 4C3 were detected and analysed as described below.

Non-denaturing polyacrylamide gel electrophoresis [14] was carried out as described in Sigma technical bulletin MKR 137, except modified for mini-slab gels. Non-denaturing gels for Coomassie blue staining were 0.75 mm thick and poured onto Gelbond-PAG (FMC, Rockland, ME) and non-denaturing gels for western blotting were 1.5 mm thick (with no Gelbond used). Following transfer, the nitrocellulose membranes were blocked in 2% (w/v) BSA (Fraction V, Sigma). C1-inhibitor was detected with rabbit antiserum against C1-inhibitor (at 1/4000 dilution) or with monoclonal antibodies 3C7 and 4C3 (at 1.5 μg/ml), and revealed with goat anti-rabbit IgG or goat anti-mouse IgG conjugated to horseradish peroxidase. Total protein staining was with an Avidin-Biotin total protein stain kit (Bio-Rad, Richmond, CA). For the Western blots involving tryptic peptides of C1-inhibitor (Table 1), the SDS gels were 10–20% (w/v) acrylamide and contained 0.1 mM thioglycolate. The proteins were transferred onto PVDF membranes, which were then blocked in 10% (w/v) dried milk/0.1% (v/v) Tween-20. These blots were detected using rabbit anti-C1-inhibitor or 4C3, peroxidase conjugated second antibody, and detected by the ECL chemiluminescence method (Amersham, Amersham, UK). For sequence analysis PVDF membranes were stained with 0.1% (w/v) Amido black/20% (v/v) methanol. The stained peptides were cut from the membrane and sequenced by the Department of Medical Biochemistry, University of Geneva.

3. Results and discussion

3.1. Formation and properties of C1-inhibitor polymers

To determine the effect of heating on the formation of polymeric C1-inhibitor species, C1-inhibitor was incubated at 50, 55, 60 and 65°C, followed by electrophoresis on non-denaturing gels (Fig. 1). At 50°C C1-inhibitor was stable for 60 min. At 55°C polymers started to appear after 5 min. At 65°C polymers were formed after 1 min, with almost no native inhibitor remaining. At the longer incubation times the size of the polymers increased. Longer incubations at 38°C showed that C1-inhibitor was stable for at least 6 days, although by 14 days C1-inhibitor was fully polymerised (not shown). Mild denaturation of C1-inhibitor by incubation with 0.9 M guanidine at 38°C induced extensive polymerisation within 24 h (not shown), as has also been shown for antithrombin [6].

Native C1-inhibitor showed two bands on the non-denaturing gels (Fig. 1, lane 1). Control experiments showed that both

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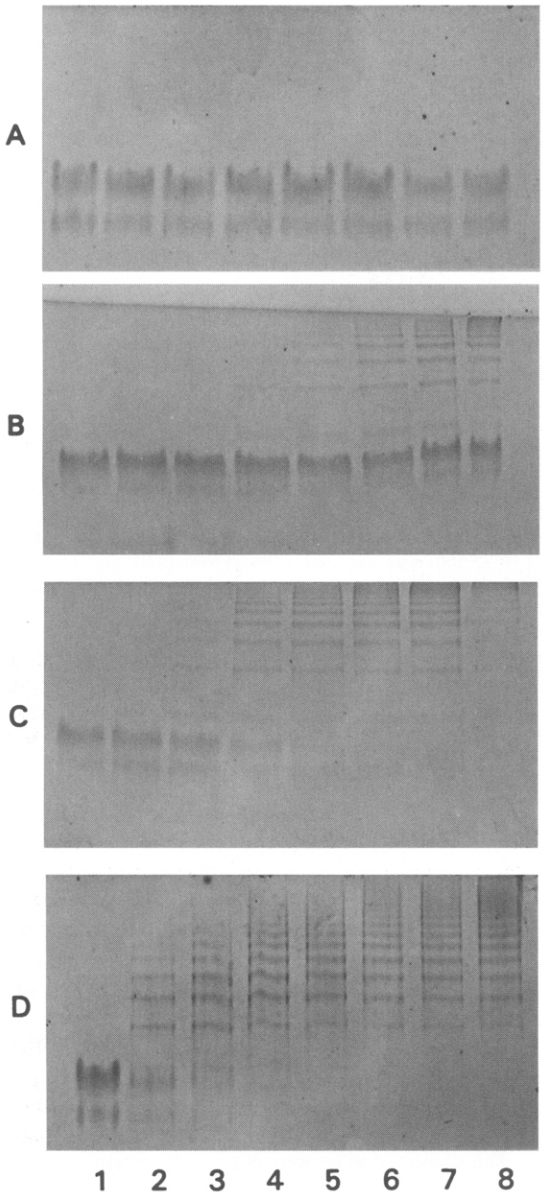

Fig. 1. Non-denaturing gel electrophoresis (4.5% (w/v) acrylamide) of C1-inhibitor incubated at various temperatures. C1-inhibitor (lane 1) was incubated at 50°C (panel A), 55°C (panel B), 60°C (panel C), or 65°C (panel D) for 1 min (lane 2), 2 min (lane 3), 5 min (lane 4), 10 min (lane 5), 20 min (lane 6), 30 min (lane 7) or 60 min (lane 8), followed by addition of non-denaturing sample buffer and storage on ice until loading on the gel. Each lane contained 5 μg of C1-inhibitor.

bands were fully active since an excess of C1s could completely complex C1-inhibitor when samples were run on SDS-PAGE. The presence of disulphide linked dimers was ruled out given that C1-inhibitor runs as one band on non-reduced SDS-PAGE. It is not clear if the two bands represent an active dimer or whether the bands are monomeric and run differently under non-denaturing conditions due to different glycosylation.

The susceptibility to degradation by trypsin of native and polymerised C1-inhibitor was compared. The primary site of cleavage by trypsin in native C1-inhibitor is the P_1–P_1' bond which produces a major fragment of 95,000 Da on non-reduced SDS-PAGE [15,16]. On SDS-PAGE both native (Fig. 2, lane 1) and heated (Fig. 2, lane 6) C1-inhibitor run as a monomer at 105,000 Da. Under the conditions used for the trypsin digestion, only a trace amount of the 95,000 Da band was produced from native C1-inhibitor (Fig. 2, lanes 2–5). Under identical conditions, the polymerised C1-inhibitor was extensively degraded to produce a major fragment of 95,000 Da and a minor fragment of 67,000 Da (Fig. 2, lanes 7–10). The 95,000 Da fragment is likely to be the P_1–P_1' bond cleaved species. Previously, however, it was shown that heated C1-inhibitor was not cleaved by kallikrein [10]. This could be because the optimal interaction of kallikrein with C1-inhibitor requires exosites which might be inaccessible or lost in the polymers. Exosites involving the P_4' residue of PAI-1 have been shown to be essential for reaction of t-PA with PAI-1 [17]. If the polymers are formed by the insertion of part of the reactive centre loop of one molecule into β-sheet C of an adjacent molecule, such that the reactive centre loop of this second molecule is disrupted and can interact with β-sheet C of another and so on [3], then the P_1–P_1' bonds might still be accessible to trypsin, and perhaps in a more favourable conformation for cleavage. In addition, when C1-inhibitor is in a large excess over trypsin, such as is the case here, a transient inhibition of trypsin can be detected by chromogenic substrate assay (Patston, unpublished data). Thus, part of the apparent difference might also reflect that polymeric C1-inhibitor is not able to inhibit trypsin.

3.2. *Immunoreactivity of polymerised C1-inhibitor*

Monoclonal antibody 4C3 binds to cleaved and complexed C1-inhibitor but not to the native protein [9,10] and is, therefore, an indicator of conformational changes involving the reactive centre loop. Thus, it was tested if polymerisation could induce the formation of the epitope. Native, heated and C1s-complexed C1-inhibitor were run on non-denaturing gels, followed by Western blotting with 3C7 and 4C3 (Fig. 3). Monoclonal antibody 3C7 detected all forms of the inhibitor [9] as can be seen from panel A, where it bound to the native doublet (lane 1), the polymers (lane 2), and the C1s-C1-inhibitor complex (lane 3). In contrast, 4C3 bound to the polymeric and complexed C1-inhibitor but not to the native protein, thereby confirming that the doublet was native protein, and indicating that the polymers had undergone a conformational change similar to that occurring on cleavage at P_1 or complexation with proteinase (panel B). For comparison, the protein stained blot is shown in panel C. The lowest molecular weight band seen with the heated C1-inhibitor (lane 2) bound to 4C3, yet ran close to the position of native protein. This probably represents

Table 1
Trypsin cleavage fragments of C1-inhibitor and reactivity with 4C3

Peptide M_r (kDa)	Reactivity with 4C3	N-terminal amino acid	Probable peptide sequence
80	+	Ile-56	Ile-56–Arg-444*
48.5	+	Val-141	Val-141–Arg-444*
38	–	Val-228	Val-228–Ala-478**
26	+	Met-288	Met-288–Arg-444*

*Arg-444 is the P_1 residue. Cleavage at this site induces the formation of the 4C3 epitope.
**Ala-478 is the C-terminal residue. This peptide does not bind to 4C3 probably because the P_1–P_1' bond is intact.

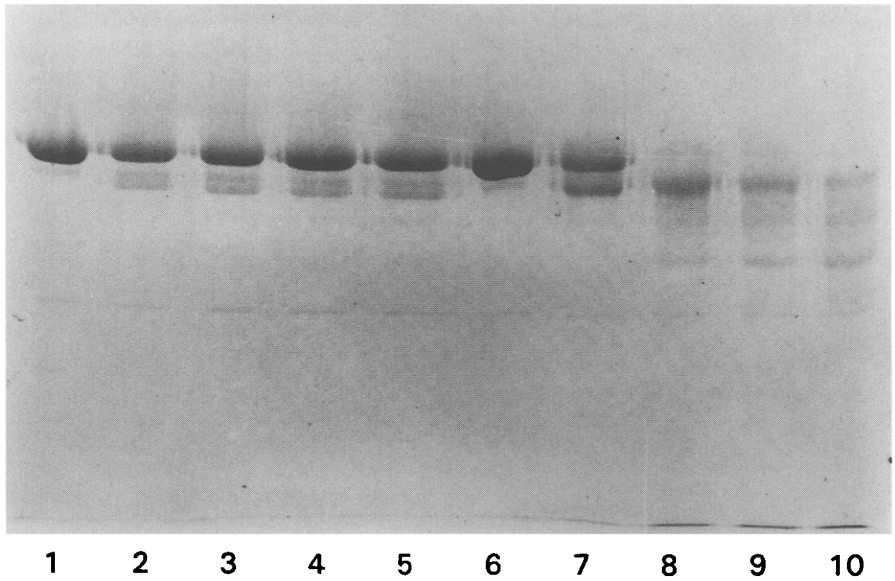

Fig. 2. Cleavage of native and polymerised C1-inhibitor by trypsin. Lane 1 = native C1-inhibitor (5 μg); lanes 2–5 = native C1-inhibitor was incubated with active site titrated trypsin at a 1:50 (w:w) ratio for 1 min (lane 2), 5 min (lane 3), 10 min (lane 4) or 20 min (lane 5), followed by addition of PMSF to 0.5 mM and DCI to 0.8 mM and sample buffer; lane 6 = heated C1-inhibitor (65°C, 30 min); lanes 7–10 = heated C1-inhibitor was incubated with active site titrated trypsin at a 1:50 (w:w) ratio for 1 min (lane 7), 5 min (lane 8), 10 min (lane 9) or 20 min (lane 10) followed by addition of PMSF to 0.5 mM and DCI to 0.8 mM and sample buffer. The samples were run on 7.5% (w/v) acrylamide SDS-PAGE.

a monomeric form in which the reactive centre loop has undergone partial insertion into β-sheet A with corresponding loss of β-sheet 1C, but without interaction with another molecule, such as is proposed to occur in the latent form of α_1-proteinase inhibitor [18].

3.3. Localisation of the epitope for monoclonal antibody 4C3

The epitope for 4C3 is present on cleaved, complexed and polymeric C1-inhibitor but not the native protein. To localise the epitope, native C1-inhibitor was extensively digested with trypsin, and subjected to electrophoresis and Western blotting. The peptides which bound to 4C3 and the amino-terminal residue of these peptides are shown in Table 1. The smallest peptide reacting with 4C3 was at 26 kDa, and amino-terminal sequencing showed that it began at Met-288. Thus, since the 4C3 epitope is produced as a result of cleavage after Arg-444, this localises the epitope to a maximum peptide length of residues 288–444, which would be consistent with the size of this fragment. This region would contain, in linear order, the secondary structure elements helix G, helix H, β-sheet 2C, β-sheet 6A, helix I, β-sheet 5A, and the reactive center loop (β-sheet 4A in the cleaved molecule) [1]. Previously it was shown that 4C3

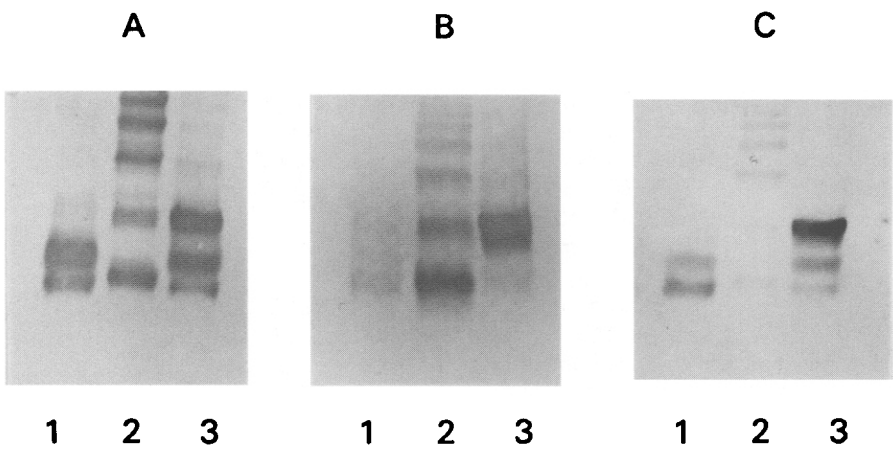

Fig. 3. Western blots of non-denaturing gels (5.5% (w/v) acrylamide) showing presence of the 4C3 epitope on polymerised C1-inhibitor. 2 μg native C1-inhibitor (lane 1), 5 μg heated (65°C, 30 min) C1-inhibitor (lane 2) and 2 μg native C1-inhibitor incubated with 3 μg C1s for 30 min (lane 3), were run on the non-denaturing gel followed by transfer to nitrocellulose paper. Panel A is C1-inhibitor detection with monoclonal antibody 3C7 (which recognizes all forms of the protein), panel B is detection with monoclonal antibody 4C3 and panel C is total protein staining.

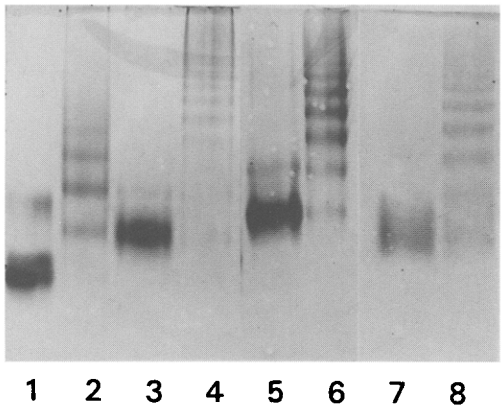

Fig. 4. Polymerisation of other serpins. α_1-Antichymotrypsin (lanes 1 and 2), α_2-antiplasmin (lanes 3 and 4), thyroxine binding globulin (lanes 5 and 6) and angiotensinogen (lanes 7 and 8) were run on 6.5% (w/v) acrylamide (lanes 1–4) or 7% (w/v) acrylamide (lanes 5–8) non-denaturing gels without heating (lanes 1, 3, 5 and 7) or after heating at 65°C for 30 min (lanes 2, 4 and 6) or 1 min (lane 8). 10 μg of protein was run in each lane.

did not bind to a synthetic peptide corresponding to residues P_{19}–P_4 [19], a region which contains part of the reactive centre loop and part of β-sheet 5A. With this information the 4C3 epitope can be localised further to residues 288–425. On the basis of the data reported here and the evidence supporting the reactive centre loop-β sheet C interaction mechanism of polymerisation [3], it seems feasible that the conformation of β-sheet 2C is an important determinant of neo-epitope expression.

3.4. Polymerisation of other serpins

C1-inhibitor, α_1-proteinase inhibitor, antithrombin III and PAI-2 all readily polymerise [4–8,20–22]. To test if this is a general phenomenon of serpins, the ability of α_1-antichymotrypsin, α_2-antiplasmin, thyroxine binding globulin and angiotensinogen to polymerise on heating was tested (Fig. 4). These serpins all formed polymers as a result of heating at 65°C. The ability of a serpin to polymerise does not correlate with inhibitory activity given that the non-inhibitory thyroxine binding globulin, angiotensinogen, and the C1-inhibitor (Mo) mutant [7] all readily polymerise. In contrast, heating or mild denaturation of the non-inhibitory ovalbumin induces the formation of the more stable *S*-ovalbumin isomer and heating of PAI-1 causes latent PAI-1 formation ([23,24]; and data not shown). *S*-Ovalbumin is probably formed by partial insertion of residues in the P_{12} region into β-sheet A [23], whereas latent PAI-1 is formed by complete insertion of the reactive centre into β-sheet A [25]. Why these serpins do not form polymers is not clear.

3.5. Conclusions

In this study we have shown that normal plasma C1-inhibitor forms polymers on heating or mild denaturation. This results in the appearance of a neo-epitope which is probably associated with β-sheet C. Although the mechanism remains speculative polymer formation is a common feature of most serpins, and can be added to biosynthesis, secretion, ligand binding and latent state formation as another mechanism by which serpin activity, and therefore extracellular proteolytic pathways, can be regulated. In addition, polymer formation is often associated with dysfunctional serpins and is clearly indicated in the pathogenesis of disease [4–7].

Acknowledgements: This work was funded by the Swiss National Science Foundation Grant 31-36080.92 (to M.S.) and the National Institutes of Health Grants HL-49242 (to P.A.P.) and Training Grant 5-T-32DK07186-18 (for support of M.M.). We wish to thank Dr. Peter Gettins for comments on the manuscript.

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